Sex chromosome evolution and speciation in stickleback fishes

HEINI M. NATRI

Ecological Genetics Research Unit
Department of Biosciences
Faculty of Biological and Environmental Sciences
University of Helsinki
Finland

ACADEMIC DISSERTATION

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Supervised by	Professor Juha Merilä Department of Biosciences University of Helsinki, Finland
	Dr. Takahito Shikano Department of Biosciences University of Helsinki, Finland
Reviewed by	Professor Deborah Charlesworth School of Biological Sciences, University of Edinburgh, United Kingdom
	Associate Professor Bengt Hansson Department of Biology, Lund University, Sweden
Examined by	Professor Judith Mank Department of Genetics, Evolution and Environment, University College London, United Kingdom
Thesis advisory committee	Professor Anssi Saura Department of Molecular Biology, Umeå University, Sweden
	Senior Lecturer Christina Nokkala Department of Biology, University of Turku, Finland
Custos	Professor Veijo Kaitala Department of Biosciences University of Helsinki, Finland

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HMN Heini M Natri
JM Juha Merilä
TS Takahito Shikano
YS Yukinori Shimada

ABSTRACT

Sex chromosomes are involved in several fundamental biological processes such as sex determination, sexual selection and dimorphism, as well as speciation. Structurally distinct sex chromosomes have evolved multiple times independently in various taxa, and different sex determination and sex chromosome systems are often found in closely related species of non-mammalian vertebrates. While the evolution of heteromorphic sex chromosomes from an ordinary pair of autosomes that obtain a sex-determining role has been hypothesized, the exact process and mechanisms of sex chromosome differentiation are not well understood. The restriction of recombination is known to be a crucial step in the process of sex chromosome evolution, and two main models have been proposed to explain the process of recombination suppression: (1) the gradual reduction of cross-overs driven by genetic modifiers of recombination rates, and (2) a stepwise model based on chromosomal rearrangements, such as inversions.

It has been hypothesized that chromosomal transitions are connected with the formation of new species. However, it is unknown whether such transitions lead to, or result from, the restriction of genetic exchange between groups that ultimately results in speciation. Sex chromosomes may have a substantial role in speciation by contributing to reproductive isolation between different taxa, particularly through hybrid sterility. Sex chromosomes are a plausible source of the effects of Haldane's rule – one the most consistent rules of evolutionary biology – which states that the heterogametic sex (XY or ZW) is more likely to exhibit hybrid incompatibility than the homogametic sex. Furthermore, genetic factors associated with reproductive isolation are often located on sex chromosomes.

In this thesis I have investigated the molecular evolution of sex chromosomes – specifically the molecular process of recombination suppression and sex chromosome differentiation – and the role of sex chromosomes in speciation by using stickleback fishes as a model system. By utilising various genetic methods, I have investigated the inter- and intraspecific variation in sex chromosome differentiation in three-spined and nine-spined sticklebacks. I have examined the neo-sex chromosomes of the Japan Sea lineage of the three-spined stickleback in order to gain insight into the process of recombination suppression and genetic differentiation of sex chromosomes at the initial stages of their evolution. Furthermore, through comprehensive breeding experiments and genetic analyses, I studied the evolution of sex determination and sex chromosome systems, as well as the genetic mechanisms of hybrid incompatibility in nine-spined sticklebacks.

The results presented in this thesis illustrate a striking variety in sex chromosome and sex determination systems between and within these stickleback species. My findings suggest that recombination suppression and genetic differentiation of sex chromosomes may develop in various ways: my results show that the differentiation process has proceeded in a gradual manner in the earliest stages of neo-sex chromosome evolution in three-spined sticklebacks, yet they demonstrate a rapid process of sex chromosome evolution within the nine-spined stickleback species driven by a chromosomal inversion. Moreover, this thesis provides evidence for the role of sex-chromosomal inversions in the formation of postzygotic reproductive isolation and thus speciation.

TIIVISTELMÄ

Sukupuolikromosomeilla ja niiden geneettisillä ominaispiirteillä on tärkeä rooli useissa keskeisissä biologian prosesseissa, kuten sukupuolen määräytymisessä, seksuaalivalinnassa, sukupuolidimorfian evoluutiossa sekä lajiutumisessa. Rakenteeltaan tunnistettavat sukupuolikromosomit ovat syntyneet evoluution myötä useita kertoja eri eliöryhmissä. Kalojen, sammakkoeläinten, lintujen ja matelijoiden luokissa jopa läheiset sukulaislajit voivat erota toisistaan sukupuolen määräytymismekanismin ja sukupuolikromosomiensa rakenteen suhteen. Heteromorfiset X ja Y (tai Z ja W) kromosomomit saavat alkunsa, kun tavalliset autosomit omaksuvat sukupuolta määrittävän roolin. Se, miten sukupuolikromosomien geneettinen eriytyminen tarkalleen tapahtuu, ei kuitenkaan ole selvää. Tärkeä tekijä eriytymisprosessissa on ns. proto-sukupuolikromosomien välisen rekombinaation estyminen. Rekombinaation estyminen voi edetä graduaalisesti tai kromosomaalisten uudelleenjärjestäytymisten, kuten inversioiden vaikutuksesta. On esitetty hypoteeseja siitä, miten rakenteelliset muutokset ja populaatioiden väliset erot sukupuolikromosomeissa voivat olla yhteydessä uusien lajien muodostumiseen. Ei kuitenkaan ole varmaa, ovatko sukupuolikromosomeissa tapahtuvat muutokset risteytyvien populaatioiden välisen geenivirran ehtymisen ja siitä seuraavan geneettisen eriytymisen ja lajiutumisen syy vai seuraus. Sukupuolikromosomit ovat yhteydessä lisääntymisisolaation, erityisesti hybridien steriiliyteen. Usein steriili sukupuoli on heterogameettinen, XY tai ZW. Lisääntymisisolaatioon vaikuttavat geneettiset tekijät sijaitsevat usein sukupuolikromosomeissa.

Väitöskirjassani olen tutkinut sukupuolikromosomien molekylaarista evoluutiota – erityisesti rekombinaation estymisen ja sukupuolikromosomien geneettisen eriytymisen prosessia – sekä sukupuolikromosomien roolia lajiutumisessa. Mallilajeina olen käyttänyt kahta piikkikalalajia, kolmipiikkiä ja kymmenpiikkiä. Useita geneettisiä analyysimenetelmiä hyödyntäen olen kartoittanut sukupuolen määräytymisen ja sukupuolikromosomien rakenteen lajienvälistä ja lajinsisäistä vaihtelua. Tutkiakseni rekombinaatiosupression ja sukupuolikromosomien eriytymisprosessia nuorten sukupuolikromosomien evoluution alkuvaiheissa, olen keskittynyt erityisesti Japaninmeren kolmipiikkien neo-sukupuolikromosomeihin. Laajojen risteytyskokeiden ja geneettisten analyysien avulla olen tutkinut sukupuolen määräytymisen ja sukupuolikromosomien evoluutiota sekä hybridien steriiliyden geneettisiä mekanismeja kymmenpiikeillä.

Väitöskirjani tulokset osoittavat, että näiden kahden tutkimuslajin sukupuolikromosomeissa on huomattavaa muuntelua, joka on syntynyt erittäin lyhyellä evolutiivisella aikavälillä. Löydökseni viittaavat siihen, että rekombinaatiosupressio ja sitä seuraava geneettinen eriytyminen etenevät graduaalisesti sukupuolikromosomien evoluution alkuvaiheissa neo-sukupuolikromosomeissa. Toisaalta tulokseni havainnollistavat kuinka sukupuolikromosomit voivat nopeasti eriytyä toisistaan kromosomaalisen inversion aikaansaamana. Väitöskirjassani esitetyt tulokset tarjoavat myös todistusaineistoa sille, että sukupuolikromosomaaliset inversiot ovat yhteydessä lisääntymisisolaation syntymiseen ja siten lajiutumiseen.

SUMMARY

Heini M Natri

Ecological Genetics Research Unit, Department of Biosciences, PO Box 65 (Viikinkaari 1), 00014 University of Helsinki, Finland

1. INTRODUCTION

1.1. EVOLUTION OF SEX CHROMOSOMES

Sex chromosomes are involved in several fundamental biological processes such as sex determination, sexual selection and dimorphism, and speciation (Charlesworth *et al.* 2005, Johnson and Lachance 2012, Dean and Mank 2014). The exceptional properties of sex chromosomes, compared to autosomes, have made sex chromosome evolution the target of countless theoretical and empirical studies. While each autosome is expected to be represented equally in males and females, sex chromosomes carried by the heterogametic sex – the Y chromosome in a male heterogametic XY and the W in a female heterogametic ZW system – face evolutionary forces that differ from those affecting autosomes due to the following factors (Charlesworth *et al.* 2005, Johnson and Lachance 2012). First, the relative rates of evolution are expected to be higher on sex chromosomes than autosomes because of their higher mutation rates (Charlesworth *et al.* 1987). Under positive selection on new, favourable mutations that on average are partially recessive, substitution rate is higher for X-linked than autosomal mutations (Charlesworth *et al.* 1987). In addition, the Y chromosome is particularly exposed to new mutations due to multiple cell divisions during gametogenesis, and the oxidizing environment in which sperm is stored (Graves 2006). Sex chromosomes experience greater effects from random genetic drift compared to autosomes due to a lower effective population size (Vicoso and Charlesworth 2006). Furthermore, genes evolve faster when they are located in chromosomes that spend more time in males (Kirkpatrick and Hall 2004). As a result, traits linked to Y- and Z-chromosomes will evolve faster than those linked to autosomes, followed by those linked to X- and W-chromosomes (Charlesworth *et al.* 1987, Kirkpatrick and Hall 2004, Qvarnström and Bailey 2009). Second, sexually antagonistic alleles as well as genes affected by sexual selection are expected to accumulate on sex chromosomes, and recombination between sex chromosomes is expected to become restricted (Bull 1983, Rice 1996, Charlesworth *et al.* 2005, Bachtrog 2006). Third, as the sex-specific chromosome degenerates, the heterogametic sex is expected to become exposed to deleterious recessive mutations, which is often associated with the Haldane's rule characterized by stronger negative fitness effects on hybrids of the heterogametic sex than of the homogametic sex (Coyne and Orr 2004). These differences between sex chromosomes and autosomes have led to the formation of genetically and morphologically distinct sex chromosomes in various groups of organisms (Bachtrog *et al.* 2014), and to a disproportionally large role of sex chromosomes in speciation, particularly speciation by genomic conflict (Johnson and Lachance 2012).

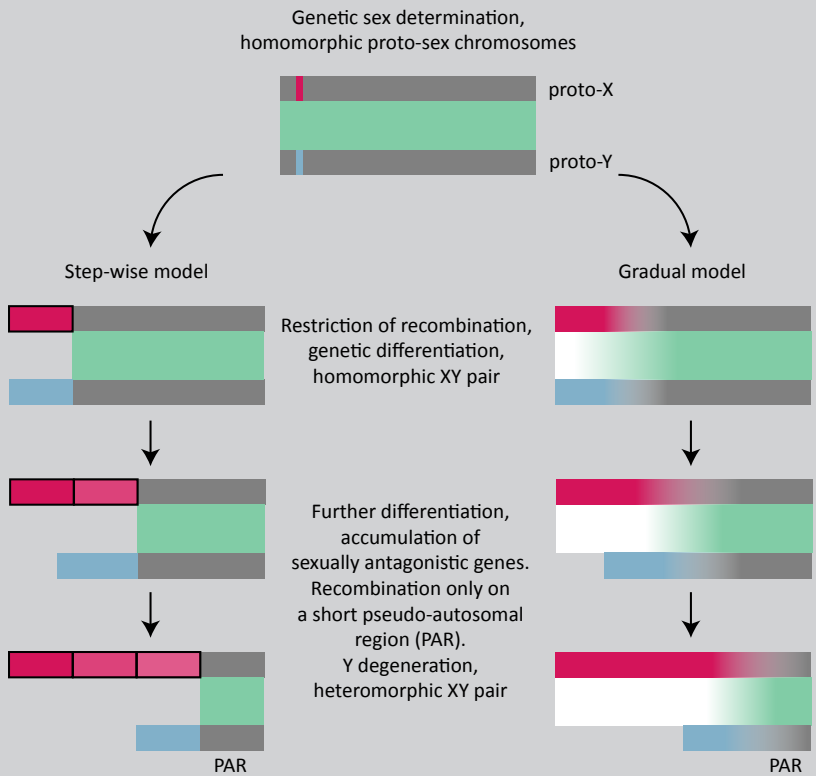
Structurally distinct sex chromosomes have evolved multiple times independently in various taxa (Charlesworth 1996, Rice 1996). Even though some well-characterized sex chromosomes, such as the mammalian XY, exhibit little variation among species, findings based on various taxa show that sex-determining mechanisms are diverse and labile, and transitions in sex determination may evolve rapidly (Bachtrog *et al.* 2014). Non-mammalian vertebrates, particularly teleost fish, exhibit a wide range of sex determination systems, from environmental sex determination to various genetic and chromosomal mechanisms (Mank and Avise 2009). This variation is partly associated with the dynamic nature of the teleost fish genomes: gene, chromosome, and genome duplications as well as chromosomal re-arrangements are strikingly common in the teleost clade (Mank and Avise 2006, 2009). Mechanisms causing transitions between heterogamety systems are not well understood, but several hypotheses have been proposed, including genetic drift, selection favouring new sex-determining alleles, sex-ratio selection, and several types of transmission distortion (van Doorn and Kirkpatrick 2007, 2010). Chromosomal transitions may play a substantial role in the evolution of chromosomal sex determination. A theoretical model illustrates that the turnover of sex chromosomes may be induced by an inversion capturing a new sex-determining mutation and a sexually antagonistic gene on an autosome, which then takes over the main sex-determining function from the ancestral sex chromosomes (van Doorn and Kirkpatrick 2007). Furthermore, inversions may induce transitions between male and female heterogamety (van Doorn and Kirkpatrick 2010). Transitions in heterogamety systems are possible if the ancestral sex chromosomes are not highly differentiated and the sex-specific chromosomes are not widely degenerated: As transitions between XY and ZW systems may result in offspring that are homozygous for the Y or W chromosome, such transitions are more likely if the chromosomes have similar gene content, but could be deleterious if the chromosomes are degenerated (Bachtrog *et al.* 2014). Thus, transitions in heterogamety are mainly observed in organisms with young sex chromosomes (van Doorn and Kirkpatrick 2010).

Theoretical studies have hypothesized the existence of a process whereby heteromorphic sex chromosomes evolve from an ordinary pair of autosomes by acquiring a sex-determining role (Ohno 1969, Rice 1996, Charlesworth *et al.* 2005, Bachtrog 2006). According to a widely accepted model, recombination between the newly formed proto-sex chromosomes first becomes suppressed over a limited region surrounding the sex-determining genes and then extends along the chromosomes – though excluding short pseudoautosomal regions – allowing the X and Y (or Z and W) chromosomes to diverge and therefore start to evolve independently. The suppression of recombination allows for the genetic differentiation of the sex chromosomes, as well as the accumulation of deleterious mutations, repetitive sequences and transposable elements on the sex chromosome that are only present in the heterogametic sex (Y in XY or W in ZW system). Ultimately, the Y or W chromosome starts to degenerate, resulting in morphologically distinguishable sex chromosomes (Rice 1996, Charlesworth *et al.* 2005). As such, the suppression of recombination is a crucial step in the evolution of genetically and structurally distinct sex chromosomes.

Two main hypotheses have been proposed to explain the process of recombination suppression: (1) the gradual reduction of cross-overs driven by genetic modifiers of recombination rates, and (2) a stepwise model based on chromosomal rearrangements, such as inversions, translocations and fusions (Fisher 1931, Charlesworth and Hartl 1978,

BOX 1. STAGES OF THE EVOLUTION OF HETEROMORPHIC SEX CHROMOSOMES

Two simplified models illustrate the stages of sex chromosome evolution from an ordinary pair of autosomes to a heteromorphic pair of sex chromosomes. The process begins with the appearance of a new sex-determining mutation. In an XY system, the proto-X chromosome harbours recessive female-promoting and male-suppressing alleles (red), as the proto-Y chromosome carries dominant male-promoting and female-suppressing alleles (blue). At this early stage, the proto-sex chromosomes are homomorphic, and recombination is suppressed only on a small region covering the primary sex-determining gene. Selection will favour linkage between sexually antagonistic genes and the sex-determining region, and ultimately the suppression of recombination will extend to cover a larger part of the chromosome pair. The expansion of the regions affected by recombination suppression and genetic differentiation may progress according to a step-wise or a gradual model. The step-wise model is characterized by evolutionary strata which vary in their levels of genetic differentiation (denoted by boxes). According to the gradual model, genetic differentiation follows the restriction of recombination without notable chromosomal rearrangements. In both cases, the progressive differentiation results in a heteromorphic pair of sex chromosomes and the degeneration of the sex-specific chromosome. In the final stages of sex chromosome evolution, recombination only persists on a small pseudo-autosomal region (PAR).



Bull 1983, Rice 1987, 1996, Brooks 1988, Charlesworth *et al*. 2005). Inversions specific to one of the homologous chromosomes are expected to suppress recombination between proto-sex chromosomes. Empirical studies on model organisms have provided support for the hypothesis suggesting a pivotal role of inversions in sex chromosome differentiation. Recombination appears to stop at different times on different parts of the chromosomes, resulting in 'evolutionary strata', i.e. chromosomal sections with different levels of divergence (Navarro *et al*. 1997, Lahn and Page 1999, Andolfatto *et al*. 2001, Lemaitre *et al*. 2009, Wilson and Makova 2009). The presence of evolutionary strata implies that recombination has stopped due to discontinuous events, such as inversions, in one of the homologous chromosomes (Lahn and Page1999). Evolutionary strata have been identified in various organisms, e.g. humans (Skaletsky *et al*. 2003, Ross *et al*. 2005, Katsura *et al*. 2012), mice (Sandstedt and Tucker 2004), and chickens (Handley *et al*. 2004, Nam and Ellegren 2008), all of which have relatively old sex chromosomes (60 to 350 My; Graves and Watson 1991, Lahn and Page 1999, Nanda *et al*. 1999). Since the sex chromosomes in these species are genetically and cytologically highly differentiated, it is not certain whether inversions are the cause of recombination suppression or occur as a consequence of ceased recombination (Charlesworth and Charlesworth 2000, Charlesworth *et al*. 2005, Wimmer *et al*. 2005, Wilson and Makova 2009). Furthermore, no data explicitly links the evolutionary strata of the mammalian sex chromosomes with any chromosomal rearrangements, and the boundaries between the strata are not as apparent as is expected if they were generated by inversions (Iwase *et al*. 2003, Charlesworth *et al*. 2005). Studies on *Silene latifolia*, a species with fairly young sex chromosomes (10 to 20 My, Filatov and Charlesworth 2002), suggest that the progression of sex chromosome differentiation could be gradual (Bergero *et al*. 2007, 2008). The gradual elimination of recombination can be driven by a chain reaction from sexually antagonistic loci near the sex-determining genes and the extension of the recombination suppression region, which then covers the linked loci (Rice 1996).

1.2. SEX CHROMOSOMES AND SPECIATION

Speciation may occur when genetic exchange between two populations is precluded by factors that are either environmental, such as geological barriers and ecological factors, or intrinsic, such as genetic factors (Noor and Feder 2006). Intrinsic factors, particularly genetic incompatibility between divergent populations, may give rise to postzygotic reproductive barriers through reduced fitness, as opposed to ecological speciation, which is associated with prezygotic reproductive isolation. This is because hybrid offspring of different species are often inviable or sterile, or characterized by abnormal and often deleterious phenotypic traits (Coyne and Orr 2004). Various studies illustrate the substantial role of sex chromosomes in speciation, particularly their role in the genetic basis of hybrid sterility and inviability (Noor and Feder 2006, Orr *et al*. 2007, Presgraves 2007, McDermott and Noor 2010). Empirical observations suggest that conflictual speciation characterized by postzygotic isolation has a notable effect on species divergence in several taxa, including plants, *Drosophila*, and vertebrates (for review see Crespi and Nosil 2013). Due to its evident negative fitness effects, postzygotic reproductive isolation may lead to selection favouring stricter mate choice in hybridizing populations, which may in turn result in further ecological divergence and the rise of prezygotic barriers. Such reinforcement of prezygotic isolation by natural selection may be particularly important in sympatry (Coyne and Orr 2004). On the other hand, prezygotic isolation may precede postzygotic barriers.

Thus, postzygotic and prezygotic reproductive barriers can be seen as part of a 'speciation continuum' (Seehausen *et al.* 2014). According to a theoretical model originally described by Bateson (1909), Dobzhansky (1937) and Müller (1942), hybrid incompatibility results from deleterious interactions between alleles from different ancestral populations that are combined in hybrids. Loci contributing to incompatibilities may reside in autosomes, sex chromosomes and/or cytoplasmic elements, and various possible mechanisms of hybrid incompatibility have been described (Noor and Feder 2006, Crespi and Nosil 2013). Sexually antagonistic selection is characterized by a conflict between optimizing male and female phenotypes. It is due to this that the effect of sex chromosomes on hybrid incompatibility is evidently larger than the effect of autosomes (Frank and Crespi 2011). Furthermore, sex chromosomes are a plausible source of the trends described by Haldane's rule – one of the most consistent rules of evolutionary biology – which states that the heterogametic sex (XY or ZW) is more likely to exhibit hybrid incompatibility than the homogametic sex (Haldane 1922). It has been proposed that X chromosomal loci have a disproportionately large effect on hybrid incompatibility ('large X effect', Masly and Presgraves 2007). Another component associated with Haldane's rule is 'faster male' evolution. Because male-expressed genes are subject to both female choice and male-male competition, these loci are expected to evolve disproportionally quickly (Wu and Davis 1993, Wu *et al.* 1996). Furthermore, studies on various taxa have shown that hybrid sterility evolves before hybrid inviability (Wu *et al.* 1996, Sasa *et al.* 1998, Presgraves 2002, Price and Bouvier 2002).

Chromosomal rearrangements, especially inversions, are known to be associated with reduced hybrid fitness and postzygotic reproductive isolation (Bush *et al.* 1977, White 1978, King 1993, Rieseberg 2001, Coyne and Orr 2004, Presgraves 2008). The mechanism connecting inversions to hybrid incompatibilities appears to be based on genes located on the inverted regions, rather than, for example, the lack of homology inhibiting chromosomal pairing (Coyne and Orr 2004, Kirkpatrick 2010). Genomic regions with fixed differences in inversions between populations may accumulate positively selected, locally adaptive alleles, as well as genes that contribute to reproductive isolation between species (Noor *et al.* 2001, 2007, Rieseberg 2001, Ortiz-Barrientos *et al.* 2002, Navarro and Barton 2003, Butlin 2005, Machado *et al.* 2007, Hoffman and Rieseberg 2008, Santos 2009). Chromosomal rearrangements associated with reproductive isolation are often located on sex chromosomes (Noor *et al.* 2001, Homolka *et al.* 2007, Ferree and Barbash 2009). However, it is uncertain whether chromosomal transitions lead to, or result from, the evolution of reproductive isolation and speciation, or if both factors are driven by an unknown third variable (Kirkpatrick 2010).

1.3. STICKLEBACKS AS A MODEL SYSTEM FOR SEX CHROMOSOME EVOLUTION AND SPECIATION

In the stickleback family (Gasterosteidae), sex determination varies: closely related stickleback species have different sex chromosome systems (e.g. Kitano *et al.* 2009, Ross *et al.* 2009), and due to their rapid turnover, their sex chromosomes are presumed to be in the early stages of evolution. The first cytological survey of the stickleback family revealed a heteromorphic XY pair in the black-spotted stickleback (*Gasterosteus wheatlandi*) and a heteromorphic ZW pair in the four-spined stickleback (*Apeltes quadracus*) (Chen and Reisman 1970). Further studies on the black-spotted stickleback have shown that the diploid chromosome number is 42 in the female, but 41 for the male, implying that the species has an X_1X_2Y sex chromosome system

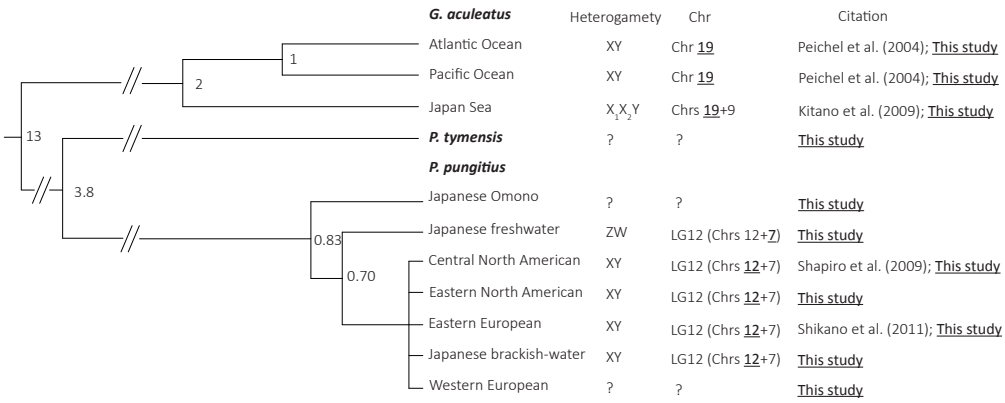


Figure 1. A summary of the sex chromosome systems of the species used in this study. Numbers on nodes refer to approximate divergence times (in My). The sex-determining gene is located on the chromosome underlined.

(Ross *et al*. 2009). Three-spined (*Gasterosteus aculeatus*) and nine-spined sticklebacks (*Pungitius pungitius*) are characterized by heteromorphic XY sex chromosomes (Ross *et al*. 2009).

Three-spined and nine-spined sticklebacks are widely distributed in the northern hemisphere. The three-spined stickleback is a popular model system in evolutionary biology (Bell and Foster 1994, McKinnon and Rundle 2002, Östlund-Nilsson *et al*. 2007), and various genomic tools have been developed to aid the research on this species (Kingsley and Peichel 2007). Since these stickleback species show similar ecological and morphological characteristics, they are suitable for comparative genetic analyses of evolutionary mechanisms (Shapiro *et al*. 2006, 2009). Phylogenetic analyses have identified genetically divergent lineages in each species. Within the three-spined stickleback, three divergent lineages have been identified – the Pacific, Atlantic and Japan Sea forms (Haglund *et al*. 1992, Higuchi and Goto 1996, Colosimo *et al*. 2005). The Japan Sea lineage diverged from the ancestor of the other two lineages approximately two Mya (Haglund *et al*. 1992, Higuchi and Goto 1996). Divergence between the Pacific and Atlantic lineages occurred approximately one Mya (O'Reilly *et al*. 1993, Ortí *et al*. 1994, Deagle *et al*. 1996). Likewise, several different lineages of nine-spined sticklebacks are identified in Europe, North America and East Asia (Takata *et al*. 1987a,b, Takahashi and Goto 2001, Aldenhoven *et al*. 2010, Shikano *et al*. 2010a). In three-spined sticklebacks, a neo-sex chromosome system formed as a result of a fusion between the ancestral Y chromosome (Chr 19) and an autosome (Chr 9) in the Japan Sea lineage after its divergence from the other (Pacific and Atlantic) lineages (Kitano *et al*. 2009). In nine-spined sticklebacks, sex is mapped to the *G. aculeatus* Chr 12, which corresponds to the nine-spined stickleback linkage group (LG) 12, which is comprised of Chr 12 and a part of Chr 7 in *G. aculeatus* (Ross *et al*. 2009, Shapiro *et al*. 2009, Shikano *et al*. 2013). Nine-spined sticklebacks carry a large Y chromosome, which has likely emerged after the species' divergence from other stickleback species (Ocalewicz *et al*. 2008). In three-spined sticklebacks, hybrid male sterility occurs in crosses between Japan Sea females and Pacific males (Yamada and Goto 2003, Kitano *et al*. 2007). Similarly, in nine-spined sticklebacks, symmetric hybrid male sterility

has been observed in crosses between two distinct lineages: Japanese freshwater and brackish-water types (Takahashi *et al.* 2005).

Interspecific variation, as well as newly formed intraspecific variation, in sex chromosome systems of these stickleback species provides an excellent model system for studying the evolutionary processes of sex chromosomes and reproductive isolation at different evolutionary time scales (Fig. 1). They allow for the assessment of inter- and intraspecific variation in the level of sex chromosome differentiation, early stages of recombination suppression and genetic differentiation of sex chromosomes, and also provide a system in which to study the role of sex chromosomes in the occurrence of postzygotic reproductive isolation between recently diverged lineages.

2. AIMS

Even though sex chromosome evolution has attracted the attention of researches for many decades, several fundamental questions regarding the process and mechanisms of sex chromosome differentiation remain unanswered. In particular, it is not clear how the cessation of recombination and the genetic differentiation of sex chromosomes progress in the early stages of their evolution (Ellegren 2011). Since direct observation of the steps and mechanisms of sex chromosome evolution is not possible, comparative analyses of closely related species and intraspecific variants with different sex chromosome systems are useful for addressing questions about the evolutionary dynamics and molecular differentiation of sex chromosomes (Rice 1996, Charlesworth and Mank 2010). In addition, neo-sex chromosomes provide excellent opportunities to study the early phases in the process of recombination suppression and sex chromosome differentiation. Neo-sex chromosomes are generated by fusions or translocations between ancestral sex chromosomes and autosomes, and thus represent the youngest examples of sex chromosomes (Nicolas *et al.* 2004, Pala *et al.* 2012a,b). Comparative analyses can be useful for assessing the time scale of sex chromosome evolution (Nicolas *et al.* 2004, Kondo *et al.* 2009, Ross *et al.* 2009, Kaiser and Bachtrog 2010, Stöck *et al.* 2011, Katsura *et al.* 2012, Pala *et al.* 2012a,b), and taxa characterized by rapid genome evolution and genome diversity can be utilised to study the chromosomal and genomic changes leading to speciation (Volff 2007). To unravel the overall patterns and process in the evolution of hybrid incompatibility, studies on species and populations along the speciation continuum are particularly valuable. Genetic mapping studies that identify the genomic locations associated with reproductive isolation in combination with comparative analyses of closely related, hybridizing groups may allow for the connection of genomic divergence and reproductive isolation. By examining populations along the speciation continuum, it may be possible to elucidate whether genomic conflicts are the cause or the result of reproductive isolation.

The purpose of this thesis was to investigate the molecular evolution of sex chromosomes – specifically the molecular process of recombination suppression and sex chromosome differentiation – and the role of sex chromosomes in speciation by using two stickleback species as model systems. To address this, the objectives of the studies included here were (1) to investigate inter- and intraspecific variation in sex chromosome differentiation in three- and nine-spined sticklebacks, (2) to examine neo-sex chromosome differentiation in three-spined sticklebacks, and (3) to study genetic mechanisms of hybrid incompatibility in the nine-spined stickleback. These objectives are addressed in the three chapters of this thesis as follows:

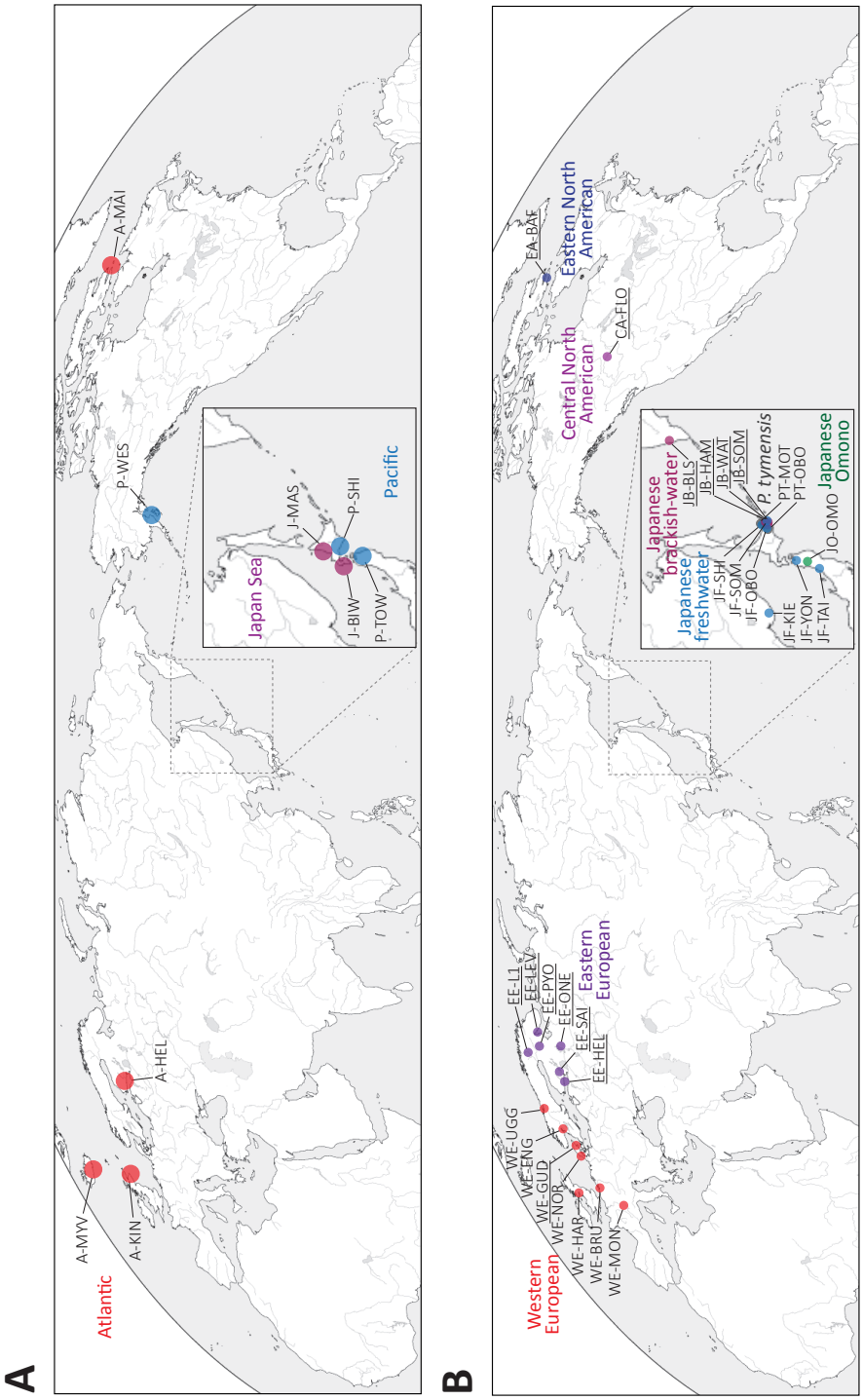


Figure 2. Sampling locations of (**A**) nine study populations of the three-spined stickleback belonging to three divergent lineages, and (**B**) 26 study populations of the nine-spined stickleback belonging to seven divergent lineages, as well as two study populations of *P. tymensis*. Populations in which male-specific alleles were observed are underlined.

Chapter **I**: The aim of the study was to examine the level and patterns of sex chromosome differentiation in three- and nine-spined sticklebacks using microsatellite markers. As stickleback sex chromosomes are thought to be in the early stages of evolution, the degree of genetic differentiation between sex chromosomes was expected to be similar in three- and nine-spined sticklebacks.

Chapter **II**: The primary objective of this study was to investigate the process and pace of recombination suppression and differentiation in the ancestral and neo-sex chromosomes of the three-spined stickleback. On the basis of these analyses, I addressed whether recombination suppression and genetic differentiation of sex chromosomes proceed according to a gradual or stepwise model.

Chapter **III**: The main objective of this study was to examine the evolutionary history and mechanisms of sex determination and sex chromosome systems as well as their connection to postzygotic reproductive isolation between recently diverged lineages of nine-spined sticklebacks. In particular, I aimed to address whether chromosomal re-arrangements are the cause or the consequence of reproductive isolation.

3. MATERIALS AND METHODS

In the following, a brief overview of the materials and methods used throughout the thesis are given. Detailed descriptions of the methods and analyses can be found in Chapters **I**-**III**.

3.1. SAMPLES

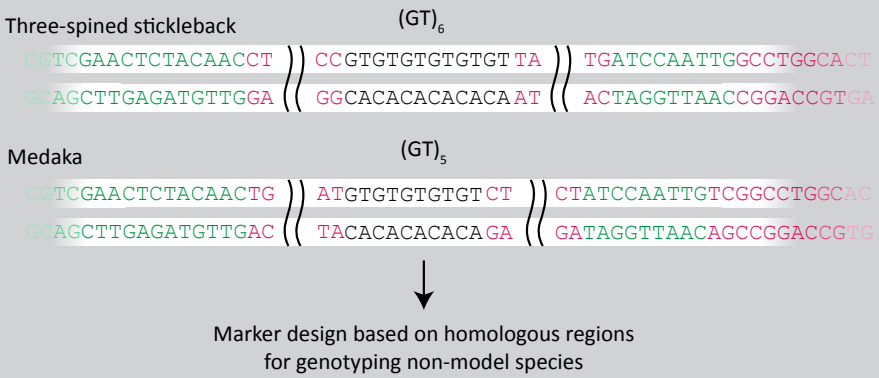
For Chapter **I**, three-spined sticklebacks were collected from the Baltic Sea, whereas nine-spined sticklebacks were collected from both the Baltic Sea (referred to as EE-HEL in Chapter **III**) and from Pyöreälampi pond (EE-PYO in Chapter **III**; Fig. 2). The two nine-spined stickleback populations were selected to cover different levels of genetic diversity (average heterozygosity at 12 microsatellite loci, H_E = 0.590 in EE-HEL and H_E = 0.004 in EE-PYO; Shikano *et al.* 2010a), which can affect the identification of sex chromosome differentiation.

For Chapter **II**, three-spined sticklebacks were collected from nine globally distributed sites (Fig. 2) and the study populations were classified into the Pacific (P-SHI, P-TOW, and P-WES), Atlantic (A-HEL, A-KIN, A-MYV, and A-MAI), and Japan Sea lineages (J-BIW and J-MAS), based on their distribution and morphological characteristics (Haglund *et al.* 1992, Higuchi and Goto 1996, Higuchi *et al.* 1996). From each population, 46 to 54 adult fish were used for genetic analyses. The genetic differentiation of sex chromosomes was examined in detail using three populations (P-SHI, A-HEL, and J-BIW) representing the three divergent lineages. For recombination and linkage analyses in the ancestral and neo-sex chromosomes of the Japan Sea lineage, three full-sib families consisting of 191 F_1 offspring were produced from the J-BIW population.

For Chapter **III**, 26 populations from phylogenetically distinct lineages of the nine-spined stickleback were utilised (Fig. 2). Based on the present and earlier studies (Takahashi and Goto 2001, Aldenhoven *et al.* 2010, Shikano *et al.* 2010a), these populations were phylogenetically classified into seven lineages, consisting of Japanese Omono (JO), freshwater (JF) and brackish-water (JB), eastern (EA) and central North American (CA), and eastern (EE) and western European (WE) lineages (Fig. 2). The three genetically and morphologically distinct forms reported in Japan – Omono, freshwater and brackish-water types (Niwa 1987, Takata

BOX 2. MICROSATELLITE PRIMER DESIGN FOR A NON-MODEL ORGANISM

Because the three-spined stickleback genome has been sequenced, microsatellite primers for this species were designed based solely on the genome sequence. Since the three- and nine-spined sticklebacks are closely related, their microsatellite loci are highly conserved (Shikano *et al.* 2010b). Therefore, the three-spined stickleback genome sequence can be utilised for primer design for the nine-spined stickleback, although amplification success is low when three-spined stickleback primers are utilised for the nine-spined stickleback (Shikano *et al.* 2010b). To improve the probability of adequate primer binding and amplification, microsatellite primers for the nine-spined stickleback were designed based on microsatellite flanking regions that are conserved (shown in green) between the three-spined stickleback genome and the genome of another sequenced model species, medaka.



et al. 1987a,b, Takahashi and Goto 2001, Tsuruta *et al.* 2002) were classified according to their morphological characteristics and confirmed with genetic analyses. The Sakhalin stickleback (*P. tymensis*) was used as an outgroup in genetic analyses.

For comparative linkage mapping and mapping of the sex-determining region, full-sib families from populations belonging to the JF and WE lineages were produced. In total, 142 individuals from three JF-YON families and 144 individuals from two WE-BRU families were used for genetic mapping analyses. For assessing the genetic conflict between the JF and WE sex chromosomes, a backcross family was produced by crossing a JF male to a female hybrid between a JF female and a WE male. F_1 hybrids were produced by artificial fertilization with a female from JF-YON and a male from WE-BRU, and a backcross was conducted using a female hybrid individual. In total, 217 backcross individuals were used to examine the suppression of recombination in the female meiosis. Similar analyses were conducted for reciprocal F_2 hybrids of the EE and WE lineages. Genetic analyses were performed using 144 individuals for the WE×EE cross and 139 individuals for the EE×WE cross. In all instances, wild fish were collected using hand nets, minnow traps or seine nets. The phenotypic sex of the sampled individuals was identified by secondary sexual characteristics and visual inspections of the gonads. All artificial crosses were carried out following procedures described in Shimada *et al.* (2011).

3.2. MOLECULAR MARKERS AND GENOTYPING

Microsatellite primers covering the ancestral and neo-sex chromosomes of the three-spined stickleback were selected and designed based on the genome sequence (Ensembl). In the initial examination of the ancestral sex chromosomes of this species in Chapter **I**, 14 microsatellite markers covering Chr 19 were used. In Chapter **II**, 63 microsatellite loci, comprised of 36 loci on Chr 19 and 27 on Chr 9, were utilised. Of these, 25 novel primer sets were developed for this study.

To study the sex chromosomes of nine-spined sticklebacks, microsatellite primers covering Chr 12 of *G. aculeatus* were designed based on conserved microsatellite flanking region sequences found in both the three-spined stickleback and medaka (*Oryzias latipes*) genomes. In addition, 46 autosomal microsatellite loci and mitochondrial cytochrome *b* sequences were used for phylogenetic analyses. To investigate the patterns and degree of sex chromosome differentiation in phylogenetically distinct lineages in the region corresponding to Chr 12, 13 populations were utilised. These included one JO (JO-OMO), three JF (JF-OBO, JF-YON and JF-TAI), two JB (JB-HAM and JB-WAT), one CA (CA-FLO), two EE (EE-HEL and EE-PYO), three WE (WE-BRU, WE-UGG and WE-NOR) and one *P. tymensis* (PT-MOT) population. In total, 613 individuals (35–52 per population) were genotyped with 35 microsatellite loci, ten of which were newly developed for this study. A total of 56 microsatellite loci for LG 12, consisting of 35 loci on Chr 12 and 21 loci on Chr 7 in the *G. aculeatus* genome, were used to genotype the parents and offspring of the mapping families, as well as the grandparents of the backcross family. Thirteen novel primer sets were designed for Chr 7.

Total genomic DNA was extracted using either a silica-based purification (Elphinstone *et al.* 2003) or a phenol-chloroform extraction method (Taggart *et al.* 1992) following a Proteinase K digestion. Each forward primer was labelled with a fluorescent dye, and PCRs were carried out using the Qiagen Multiplex PCR Kit. For Chapters **I** and **II**, PCR products were visualized using a MegaBACE 1000 automated sequencer and ET-ROX 550 size standard. Alleles were scored using Fragment Profiler 1.2. For Chapter **III**, alleles were visualized with an ABI 3730 sequencer and the GeneScan 500 ROX size standard and scored using GeneMapper 5.0.

3.3. KARYOTYPING

Karyotype analyses were conducted for the JO, JF, JB, EE, and WE lineages, as well as for *P. tymensis* according to a protocol modified from Ross and Peichel (2008). Metaphase spreads were prepared from primary epithelial cells using the wild-caught fish or lab-reared F_1 progeny of PT-MOT, JO-OMO, JF-YON, JB-HAM, EE-HEL, WE-BRU, and WE-UGG populations. Fish were injected intraperitoneally with 0.5% colchicine solution at a dose of 2 µl/g of body weight and maintained in an aquarium for four hours. Gill and spleen tissues were homogenized and incubated in 0.075 M KCl solution for 30 min on ice. Cells were fixed with Carnoy's solution (methanol:acetic acid = 3:1), placed on glass slides, and stained with 5% Giemsa solution. Metaphase chromosomes were examined using at least three females and three males from wild individuals or different full-sib families in each population.

3.4. COMMON DATA ANALYSES

Since a large proportion of microsatellites are conserved between three-spined and nine-spined sticklebacks (Shapiro *et al.* 2009, Shikano *et al.* 2010b), microsatellite locations on LG 12 in nine-spined sticklebacks were estimated based on the three-spined

stickleback genome. To evaluate genetic differentiation between sex chromosomes, F_{ST} values between males and females and their significance were determined using Genepop 4.2 (Rousset 2008). In addition, sex-specific alleles were investigated by comparing allele and genotype frequencies between females and males in each population. The number of polymorphic loci, observed and expected heterozygosities, and F_{IS} were calculated using FSTAT 2.9.3 (Goudet 1995). The presence of sex-specific null alleles was tested using MICRO-CHECKER (van Oosterhout *et al*. 2004). For Chapters **I** and **II**, linkage disequilibrium between all pairs of loci was tested in each population using Genepop 4.0 (Rousset 2008). The statistical significance of estimates was determined with consideration to multiple comparisons (Rice 1989).

For Chapters **II** and **III**, phylogenetic relationships were constructed with the Neighbor-Joining (NJ) method and 1,000 bootstrap replicates across loci using Populations 1.2.31 (Langella 2002). Phylogenetic analyses based on the X and Y chromosomal alleles identified in males were used to address whether the ancestral sex chromosomes of the three-spined stickleback differentiated from each other genetically before or after the divergence of the three lineages. Similarly, the origin of genetically distinct X and Y chromosomes in the XY diverged populations was examined in the nine-spined stickleback, as well as whether the XY chromosome pairs of the three XY diverged lineages were formed in a single event before their divergence or by multiple independent events after the initiation of their divergence.

For Chapter **III**, phylogenetic analyses were conducted for 24 globally distributed populations, including two *P. tymensis* populations, based on autosomal microsatellite loci and mitochondrial cytochrome *b* sequences. In total, 762 individuals (8–49 per population) were genotyped. The mitochondrial phylogeny was investigated based on an 1104-bp region of cytochrome *b*, which corresponds to 97% of the gene, with 117 individuals (3–10 per population). A total of 20 individuals (four from JF-KIE, five from JB-BLS, five from CA-FLO, one from EE-HEL, and five from WE-BRU) were sequenced using two primer sets with BigDye Terminator v1.1 as described in Shikano *et al*. (2010a). The sequences of the remaining individuals were acquired from earlier studies (Shikano *et al*. 2010a, Wang *et al*. 2015). Phylogenetic relationships, as well as divergence times, were inferred using the Bayesian Markov chain Monte Carlo (MCMC) method implemented in BEAST 1.8.2 (Drummond *et al*. 2012). For divergence time estimation, cytochrome *b* sequence data of the three-spined stickleback was included in the analyses.

3.5. LINKAGE MAPPING

For Chapter **II**, recombination and linkage analyses for the Japan Sea lineage were conducted for female and male meioses using JoinMap 4.1 (Stam 1993, Van Ooijen 2006). Pairwise recombination frequencies were calculated for each of the three study families and linkages between loci were detected based on these estimates. Locus order and distances in consensus linkage maps were determined by the mean recombination frequencies and combined logarithm of the odds (LOD) scores of the three families using the regression mapping approach. The Kosambi mapping function was used to convert recombination frequencies in order to map distances in cM. Recombination rates were given in cM/Mb based on the genetic and physical distances between neighbouring loci. Recombination frequencies in a pseudoautosomal region were calculated for female and male meioses based on map distances following Otto *et al*. (2011).

For Chapter **III**, linkage maps were constructed using CRI-MAP 2.5 (Green *et al*. 1990). LOD scores were calculated for all pairs of loci. The most likely order of loci with LOD scores ≥3 was determined based on multipoint linkage analysis. Consensus maps for the JF and WE lineages were generated using multiple mapping families.

3.6. QTL MAPPING

While a chromosomal region of LG 12 was identified to be responsible for the male heterogametic sex determination in the JB, EE, CA, and EA lineages, sex-specific allelic patterns were not detected in any loci in other lineages. To assess if LG 12 is responsible for sex determination in the JF and WE lineages, from which the X and Y chromosomes were indicated to have originated, association and mapping analyses were conducted with the mapping families of these lineages, as well as other crosses. First, associations of phenotypic sex with maternally and paternally segregating alleles were examined separately to identify the sex-determining region and heterogametic sex based on Fisher's exact test with standard Bonferroni correction. Second, QTL mapping was performed based on the Haseman-Elston regression method (Haseman and Elston 1973) with the Visscher-Hopper correction (Visscher and Hopper 2001) using GridQTL (Seaton *et al*. 2006). Significant QTLs were identified using the sib-pair model based on the 5% experiment-wide threshold of F statistics with 10,000 bootstrap permutations, and 95% confidence intervals of QTL loci were estimated with 10,000 replicates. Genetic maps of the heterogametic sex were used for the mapping analyses. In the crosses where no association with sex was identified, the sex-averaged maps were utilised.

Mapping analyses were also conducted for the gonad index (ratio of testes weight to body weight, expressed as a percentage; DeVlaming *et al*. 1982) and sterility (presence or absence of sperm) in the male individuals of the backcross between the JF and WE lineages under the same conditions and criteria as those used for sex. Sterile and fertile individuals were classified based on the absence and presence of sperm, respectively. Out of the 105 males of the backcross family, 90 mature individuals with nuptial coloration were utilised for the mapping analyses. The effects of tank, clutch, and body weight were not considered in the analyses, since there was no association between these factors and gonad index (Kruskal-Wallis tests for tank and clutch, Spearman rank correlation test for body weight, $P > 0.05$) or sterility (χ^2-test for tank and clutch, Mann-Whitney U test for body weight, $P > 0.05$). The mapping analyses were conducted using the female genetic map, as the segregation of the JF and WE alleles was expected to occur in the maternal meiosis of the backcross family. In addition to the mapping analyses, the proportion of sterile individuals was calculated for each genotypic combination of grandparental JF and WE alleles at loci across LG 12.

4. RESULTS AND DISCUSSION

4.1. GENETIC DIFFERENTIATION OF STICKLEBACK SEX CHROMOSOMES

In the three-spined stickleback, male-specific alleles were observed in loci closely linked to the IDH gene, which is known to be associated with phenotypic sex in this species (Avise 1976, Withler and McPhail 1985). Male-specific null alleles were identified at several loci in two different chromosomal regions (Chapter I), suggesting that the Y chromosome is highly degenerate in three-spined sticklebacks. Sex chromosomes of the nine-spined stickleback were found to be highly differentiated throughout most of their length (Chapter I). In contrast to the three-spined stickleback, signatures of Y

chromosome degeneration were not detected in the nine-spined stickleback, indicating that the Y chromosome of this species has not degenerated as much as that of the three-spined stickleback. No loci exhibited patterns of multilocus amplification, suggesting that the large Y chromosome observed in the nine-spined stickleback is not due to Y chromosome duplication. Since closely related stickleback species exhibit a rapid turnover of sex chromosomes and sex determination systems, their sex chromosomes are thought to be in the early stages of evolution (Ross *et al.* 2009). Nevertheless, high levels, but distinct patterns, of sex chromosome differentiation were observed in closely related stickleback species (Chapter I) that diverged approximately 13 Mya (Bell *et al.* 2009).

Intraspecific variation in the genetic differentiation of sex chromosomes in these species was further examined in Chapters **II** and **III**.

4.2. EVOLUTION OF THE ANCESTRAL AND NEO-SEX CHROMOSOMES OF THE THREE-SPINED STICKLEBACK

Ancestral sex chromosomes of the three-spined stickleback were found to be highly differentiated from each other throughout most of their length in all three divergent lineages (Fig. 3). In addition, allelic patterns at many loci were identical among these lineages, suggesting that the contemporary genetic differentiation and organization of the ancestral sex chromosomes was formed mainly before the divergence of the Japan Sea lineage and other lineages (Chapter **II**). Phylogenetic analyses based on X and Y chromosomal alleles support the scenario that the ancestral sex chromosomes diverged extensively before the split of these lineages, implying that the genetic differentiation detected throughout the chromosomes evolved within 2 My (Fig. 3).

Although the genetic organization of the ancestral sex chromosomes is highly conserved among the divergent lineages, different sex-specific allelic patterns were identified in some of the loci located in the central region of Chr 19 (Fig. 3). All deviant patterns were associated with the difference in Y chromosomal amplification between the Japan Sea lineage and other lineages. Cytogenetic analyses of the Pacific lineage indicated multiple inversions in the same region of the Y chromosome (Ross and Peichel 2008), suggesting that ancestral Y chromosome rearrangements might have occurred differently between the Japan Sea lineage and other lineages in this genomic region. Moreover, degeneration of the Y chromosome might have occurred differently in this region, and the rearrangements and degeneration of the ancestral Y chromosome in the Japan Sea lineage may have been affected by the formation of neo-sex chromosomes. The results also indicated varying levels of genetic differentiation between the ancestral sex chromosomes, which may have resulted from the Y chromosome inversions observed on the same region (Ross and Peichel 2008).

Recombination between the ancestral sex chromosomes of the Japan Sea lineage was extensively suppressed. Recombination frequency during male meiosis was not only reduced in the sex-determining region but was also increased in the pseudo-autosomal region of these chromosomes (Fig. 4). A possible explanation for the increased recombination rate in the heterogametic sex is that at least one crossover is required for the proper segregation of the sex chromosomes during meiosis in many species, and that recombination occurs exclusively in a small pseudo-autosomal region in the heterogametic sex (Rouyer *et al.* 1986, Soriano *et al.* 1987). Recombination frequency in the pseudo-autosomal region was estimated to be 4.6 times higher in male meiosis than in female meiosis in the three-

spined sticklebacks of the Japan Sea lineage. In contrast, the recombination rate was 1.3 times higher in males compared to females of the Pacific lineage (Otto *et al.* 2011). The rate difference observed between these lineages could be due to the extensive recombination restriction across the ancestral and neo-sex chromosomes in the Japan Sea lineage caused by the formation of a neo-sex chromosome system.

Distinct levels and patterns of genetic differentiation were found in both the ancestral and neo-sex chromosomes of the Japan Sea lineage (Fig. 4). While the ancestral sex chromosomes were highly differentiated over most of their length, loci with sex-specific patterns were only observed on a small region at one end of the neo-sex chromosomes. No male-specific null alleles were detected, providing no indication of Y-degeneration. In the region where male-specific alleles were

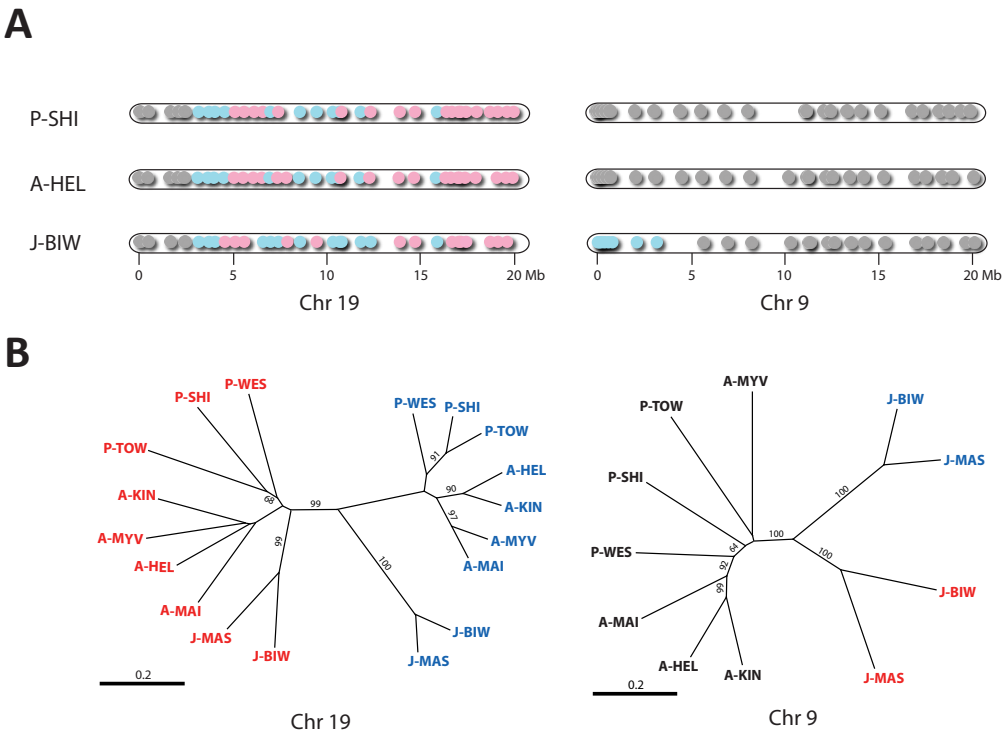


Figure 3. (**A**) Patterns of genetic differentiation in the ancestral (Chr 19) and neo-sex (Chr 9) chromosomes of the three divergent lineages of the three-spined stickleback. Locus positions are indicated in the physical maps. The blue colour represents the loci where male-specific alleles were observed, and the red colour indicates the loci where no heterozygous males were found. (**B**) Phylogenetic trees based on D_{CE} distances for X (red) and Y (blue) chromosomal alleles at 15 loci on Chr 19 and at eight loci on Chr 9 in nine study populations of the three-spined stickleback. Data from males are indicated in black. Bootstrap support values above 60% are indicated. Population names starting with P belong to the Pacific lineage, A belong to the Atlantic lineage, and J belong to the Japan Sea lineage. For population abbreviations, see Chapter II.

identified, the level of genetic differentiation was highest at the fusion point with the ancestral sex chromosomes and decreased with increasing distance from the fusion point. This suggests that the genetic differentiation was initiated at the fusion point and was then followed by gradual expansion to the neighbouring regions. In the neo-sex chromosomes, the region where recombination was suppressed was larger (7.0 Mb) than the region where genetic differentiation was observed (3.2 Mb; Fig. 4). Furthermore, recombination was reduced over a span only covering 15.4 Mb. Since genetic differentiation of sex chromosomes occurs after recombination suppression (Bengtsson and Goodfellow 1987), it appears that the differentiation of the neo-sex

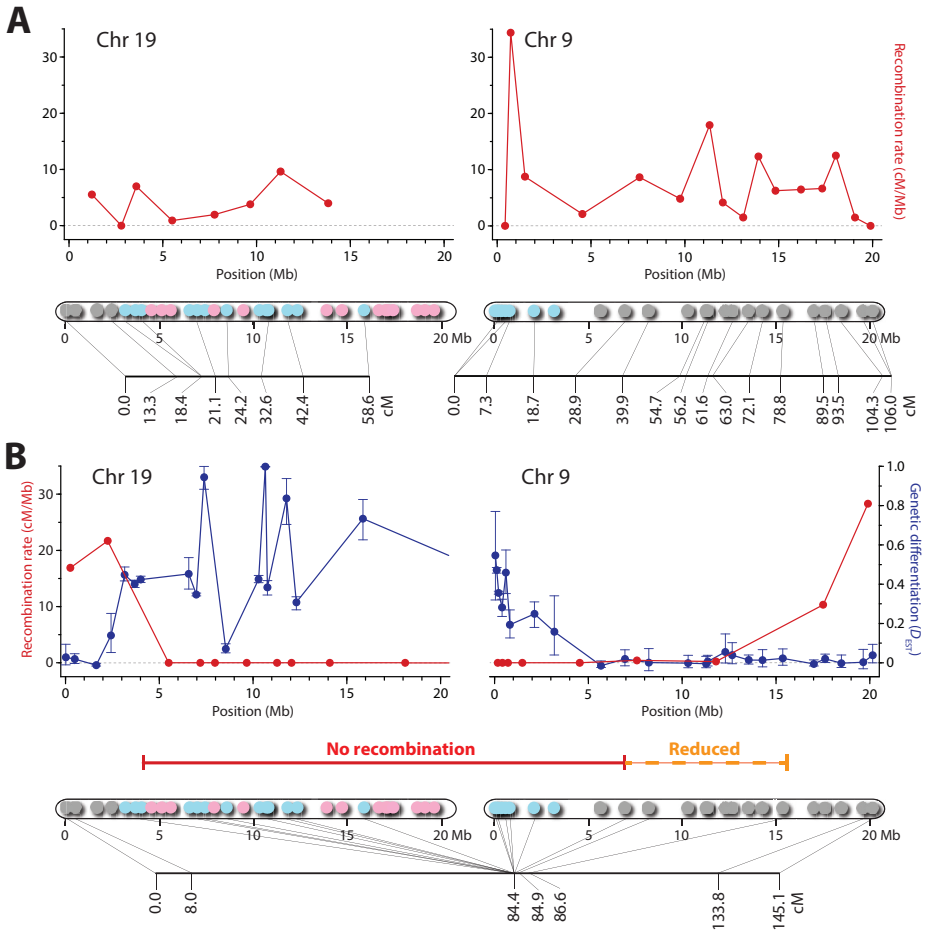


Figure 4. Recombination rates (red) and genetic linkage maps for Chrs 19 and 9 in female meiosis (**A**) and male meiosis (**B**) in the J-BIW lineage of the three-spined stickleback. D_{EST} values and 95% confidence intervals (blue) are shown in (**B**). Recombination rates are plotted against intermediate physical locations between adjacent loci. The numbers in the linkage maps represent genetic map distances in Kosambi centimorgans (cM). The colours in the physical maps indicate allelic patterns at 55 loci on Chrs 19 and 9 in J-BIW. Light blue indicates loci where male-specific alleles were identified, and pink indicates loci where male-specific null alleles were observed.

chromosomes has gradually spread from the end where the neo-Y chromosome fused to the ancestral Y, following the extension of recombination suppression. These results suggest that the progression of differentiation between the neo-sex chromosomes of the three-spined stickleback is gradual, rather than a consequence of large inversions or other chromosomal rearrangements.

4.3. DIFFERENT SEX DETERMINATION SYSTEMS IN THE NINE-SPINED STICKLEBACK

In globally distributed population samples, high levels of XY differentiation were observed in populations of nine-spined sticklebacks belonging to the JB, EE, CA, and EA lineages (Chapter **III**, Figs. 5 & 6A). These XY diverged lineages are also characterized by an exceptionally large Y chromosome (Fig. 6A). In contrast, none of the loci exhibited sex-specific allelic patterns in the JO, JF, and WE lineages, or in closely related sister species *P. tymensis*, nor were heteromorphic sex chromosomes identified in these lineages.

Based on QTL mapping results, it is revealed that both the ZW and XY sex determination systems exist within the nine-spined stickleback. In the F_2 cross between a WE female and an EE male, phenotypic sex was perfectly associated with the paternal alleles at the loci on *G. aculeatus* Chr 12 where recombination is suppressed between the X and Y chromosomes. The sex-determining gene was mapped to the region of suppressed recombination in the male map (Fig. 7), as in earlier studies of EE and CA populations (Shapiro *et al.* 2009, Shikano *et al.* 2013). However, in the JF lineage and the backcross between the JF and WE lineages, significant association with sex was found for the maternal alleles at loci on *G. aculeatus* Chr 7. This indicates that the JF lineage has a ZW sex determination system. The sex-determining gene was mapped to a region at 19.4 cM

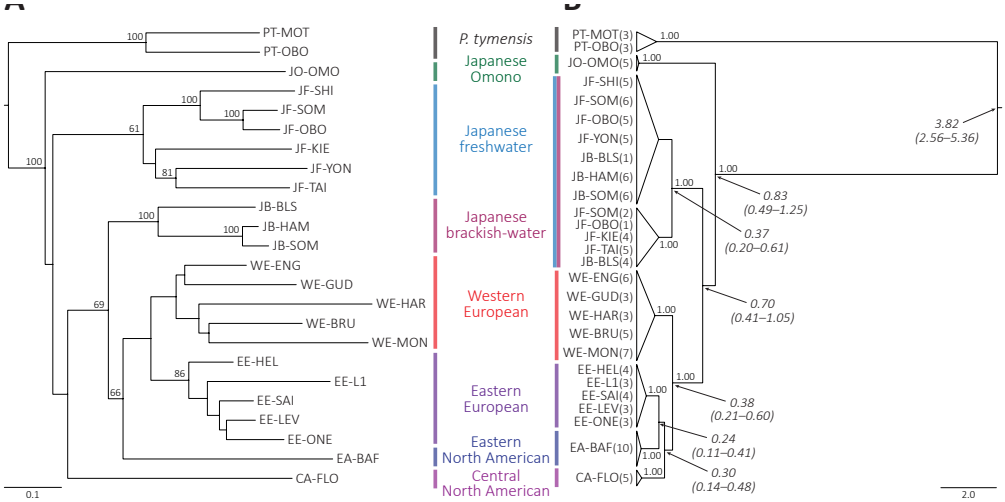


Figure 5. Phylogenetic relationships among nine-spined stickleback populations. (**A**) Phylogenetic relationships based on 46 autosomal loci. Bootstrap values (>60) are shown above the nodes. (**B**) Mitochondrial phylogeny based on cytochrome *b*. Posterior probabilities (>0.95) are indicated above the nodes. The number of individuals observed in each clade is given in parentheses after the population code. Divergence time estimates are shown in italics with the 95% highest posterior density interval in parentheses. Divergence time is given in millions of years.

in the female map of the JF lineage (276.6 cM) and a region at 15.0 cM in that of the backcross (129.5 cM; Fig. 7). No significant association with sex was identified in any of the loci across LG 12 in the mapping families of the WE lineage or in the F_2 cross between an EE female and a WE male. Phylogenetic, principal coordinate and hybrid index analyses indicate the X and Y chromosomes of the three XY diverged lineages originate from the WE and JF lineages, respectively (Fig. 6B). This implies that the XY chromosome pair was formed by a hybridization event before the divergence of the XY lineages, which was estimated to have occured ca. 0.3 Mya (Figs. 5 & 6B). As the sex-determining gene in the ancestral lineages is not found in the sex-determining region of the XY lineages, it appears that the XY system emerged in this region after the formation of the new chromosome pair. Comparative mapping of the ancestral lineages revealed a large inversion in the WE lineage in comparison to the ancestral standard arrangement exhibited in the three-spined stickleback and the JF lineage. The inversion region corresponds to the sex-determining region of the XY lineages, and when heterozygous, recombination on the inversion region becomes restricted, as shown by interlineage crosses (Fig. 7). Thus, it appears that recombination between the X and Y chromosomes of the XY lineages is suppressed due to the inversion. Suppression of recombination is critical in the evolution of sex chromosomes because it leads to the accumulation of repetitive sequences and deleterious mutations in sex-specific chromosomes (Charlesworth *et al.* 2005). In fact, in line with this, an accumulation of heterochromatin is found on the Y chromosome in the EE lineage (Ocalewicz *et al.* 2008). Therefore, the Y chromosome of the XY lineages might have been enlarged by the accumulation of repetitive elements in the non-recombining region, as is expected for sex-specific chromosomes in their early evolutionary stages prior to the initiation of degeneration (Steinemann and Steinemann 2005, Kubat *et al.* 2008, Bernasconi *et al.* 2009). It is generally

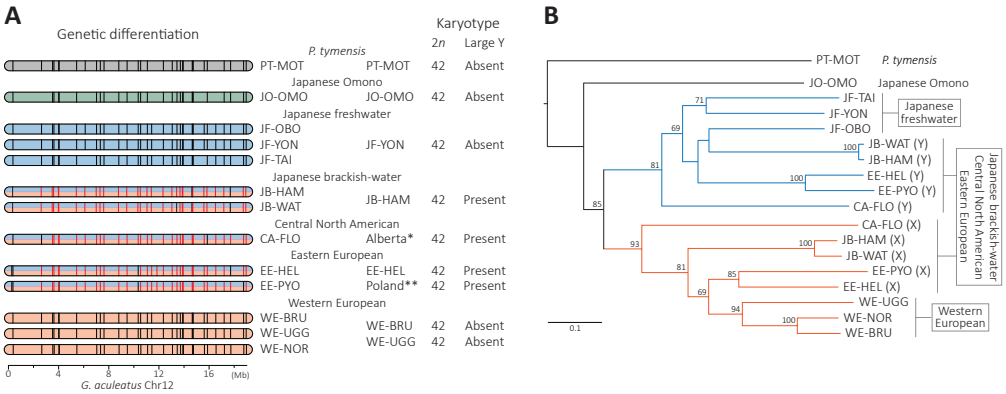
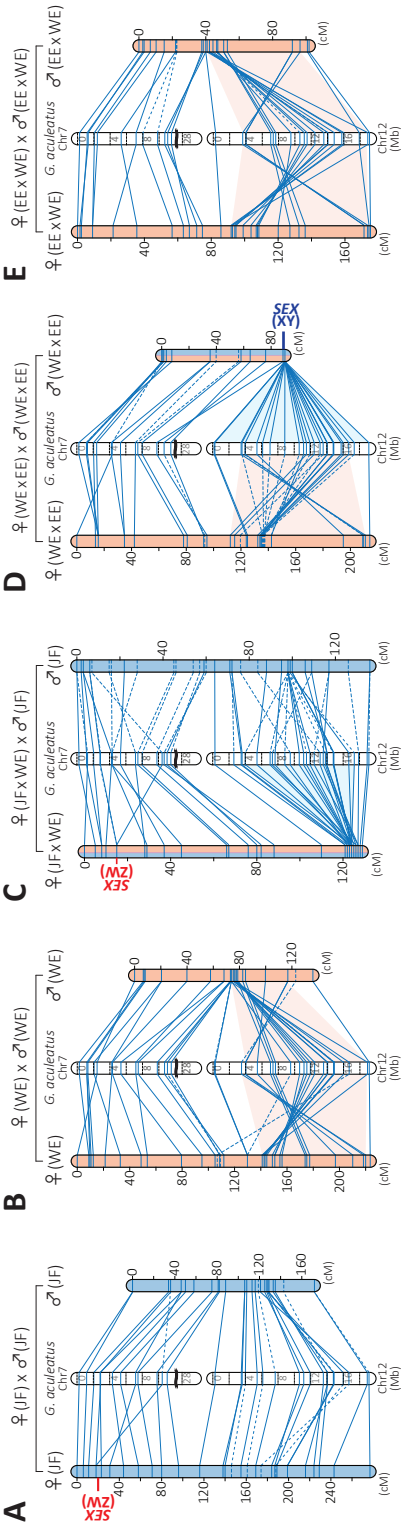


Figure 6. Genetic differentiation and evolutionary relationships among the X and Y chromosomes from different lineages of the nine-spined stickleback. (**A**) Sex-specific genetic differentiation in the region of *G. aculeatus* Chr 12 and the presence or absence of a large male-specific chromosome. Loci with male-specific alleles are indicated in red. Karyotype data for populations from Alberta and Poland are based on Ross *et al.* (2009)* and Ocalewicz *et al.* (2008)**, respectively. (**B**) Phylogenetic relationships based on *G. aculeatus* Chr 12 loci. X and Y chromosomal haplotype data are used for three lineages with the XY system. Bootstrap values (>60) are indicated above the nodes.



hypothesized that highly differentiated, heteromorphic sex chromosomes evolve from a pair of ordinary autosomes as a result of the suppression of recombination between them and the degeneration of the sex-specific chromosome (Charlesworth *et al.* 2005). In contrast, our study suggests that genetically and morphologically distinct sex chromosomes can be formed rapidly through the hybridization of ancestral lineages with different inversion states, providing an alternative view on the evolutionary trajectory of sex chromosomes.

4.4. PATTERNS AND GENETICS OF POSTZYGOTIC REPRODUCTIVE ISOLATION

Most or all F_1 hybrids were males in crosses between JF males and JB, EE, or WE females, whereas sex ratios were not biased in the F_1 hybrids of the reciprocal cross or those of other cross combinations. All hybrid males of reciprocal crosses between the JF and JB, EE, or WE lineages, and a part (45.5–84.6%) of hybrid males of reciprocal crosses between the JO and JB, EE, or WE lineages were found to be sterile (Table 1). In *P. tymensis* hybrids, male sterility was observed in crosses with JB, EE, and WE, but not in crosses with the JF lineage. As in a previous study on hybrids

Figure 7. Comparative genetic mapping and sex determination systems in the nine-spined stickleback. Comparative linkage maps with the *G. aculeatus* genome in the JF (**A**), and WE (**B**) lineages, a backcross between a JF/WE female and a JF male (**C**), and F_2 crosses between a WE female and an EE male (**D**) and between an EE female and a WE male (**E**). Different chromosome colours represent different origins, indicated by phylogenetic analyses (see Fig. 6B). Dashed lines in the comparative maps indicate uninformative (i.e. non-segregating) loci in the corresponding sex. QTL positions for sex are shown on the female maps of the JF lineage, the backcross, and on the male map of the WE×EE F_2 family.

between the JB and JF lineages, male sterility was characterized with normal testis size but a defect in spermatogenesis (Takahashi *et al*. 2005). No hybrid female sterility was identified in any cross combination. Likewise, no significant hybrid inviability in the early developmental stages was found in any of the crosses.

Despite the absence of reproductive barriers between the nine-spined stickleback and the Ukrainian stickleback (*P. platygaster*), which diverged more than 2 Mya (Ziuganov and Gomeluk 1985), it was observed that hybrid sterility is found between more recently diverged lineages of nine-spined sticklebacks. This indicates that reproductive isolation has rapidly evolved within this species. Furthermore, hybrid offspring of crosses between *P. tymensis* and the JF lineage, which diverged from each other more than 3 Mya, were found to be fertile. Since no hybrid sterility is induced in crosses between the JF and JO lineages, or among the JB, EE, and WE lineages, it is likely that shared genetic factors are at least partially involved in the incidence of sterility in different crosses. Based on the incidence of sterility, genetic factors causing hybrid sterility might have been fixed differently between the JF and WE lineages after divergence from a common ancestor, followed by a transition to the XY lineages during their formation by hybridization between the ancestral lineages on secondary contact.

To investigate whether postzygotic isolation was caused by the large inversion in the sex chromosomes, genetic mapping was conducted for gonad index and sterility in the males of the backcross between the JF and WE lineages using fertile F_1 hybrid females from a cross between a JF female and WE male parent. In the backcross generation, 76.7% of the individuals were sterile (Fig. 8A). While no significant QTL was identified for gonad index, a significant QTL for sterility was found at the 127.6 cM region of the female map (Fig. 8B). The QTL region for sterility corresponds to a region

Table 1. Male fertility percentage (%) based on sperm count in F_1 hybrids of reciprocal crosses among five divergent lineages of the nines-spined stickleback as well as F_1 hybrids of closely related *P. tymensis* (PT) males and *P. pungitius* females. For lineage abbreviations, see the Materials and Methods section.

		Female parent				
		EE	WE	JB	JF	JO
Male parent	EE	100	100	100	0	40.0
	WE	100	100	100	0	25.0
	JB	100	100	100	0	54.5
	JF	0	0	0	100	100
	JO	16.7	50.0	15.4	100	100
	PT	0	0	0	100	na

of suppressed recombination where the large inversion was found in the WE lineage. While all individuals with a combination of both WE and JF grandparental alleles in this region were sterile, the occurrence of sterility was 52.6% in individuals with only JF alleles (Fig. 8C). In addition, in the latter group, the proportion of sterility differed slightly but significantly between individuals with alleles from the different paternal grandparents in this region. Hence, sterility is largely determined by allelic combinations in the inverted region and mainly occurs when this region is heterozygous. These results provide clear evidence for the role of the inversion in the evolution of postzygotic reproductive isolation in recently diverged lineages of the nine-spined stickleback.

In the XY lineages, a new male heterogametic sex determination system has evolved in the chromosomal region involved in hybrid sterility (Fig. 9). In reciprocal crosses of the JF and WE lineages, full male sterility was observed, and in F_1 hybrids between WE females and JF males, only phenotypically

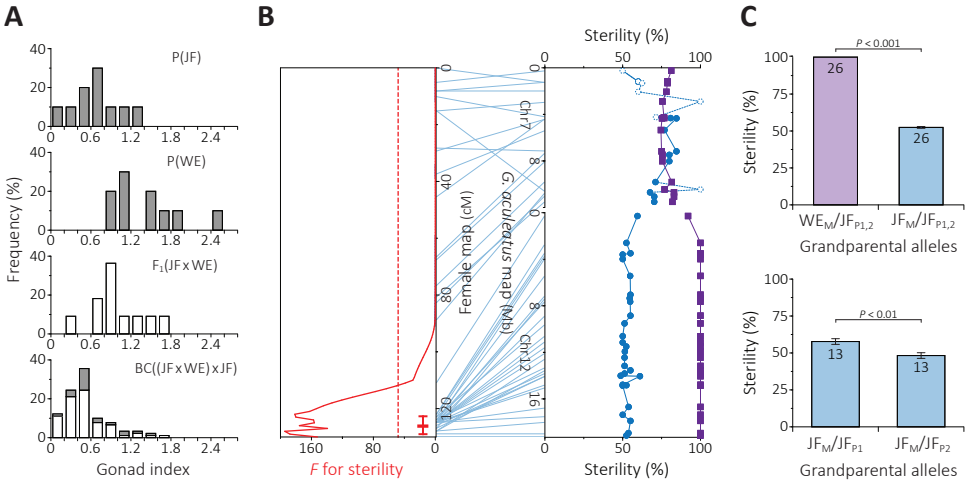


Figure 8. QTL region responsible for hybrid sterility in the nine-spined stickleback. (**A**) Frequency distributions of gonad index in the males of the JF and WE lineages, as well as in the F_1 hybrids and backcross individuals. Grey and white bars represent fertile and sterile individuals, respectively. (**B**) QTL analysis for sterility (red) and the occurrence of sterility in individuals with different allelic combinations among the backcross males. In the graph on the left, the dashed line indicates statistically significant levels (P = 0.05) of the *F*-value. The mean and 95% confidence interval of the QTL position for sterility are indicated near the map position. In the graph on the right, individuals with both WE and JF alleles are indicated in purple, and those with only JF alleles are indicated in blue. Open symbols represent data from ten or less individuals due to the presence of female-linked alleles. (**C**) The occurrence of sterility in individuals with different allelic combinations in the region where a large inversion is indicated (3.5–18.9 Mb of *G. aculeatus* Chr 12). The means and standard errors are obtained across loci in the inversion region for individuals with WE/JF and JF/JF grandparental alleles (in the upper graph) and those with JF alleles from the maternal grandmother and from the different paternal grandparents (in the lower graph). The numbers in the bars represent the numbers of loci analysed. P1 and P2 indicate different paternal grandparental alleles.

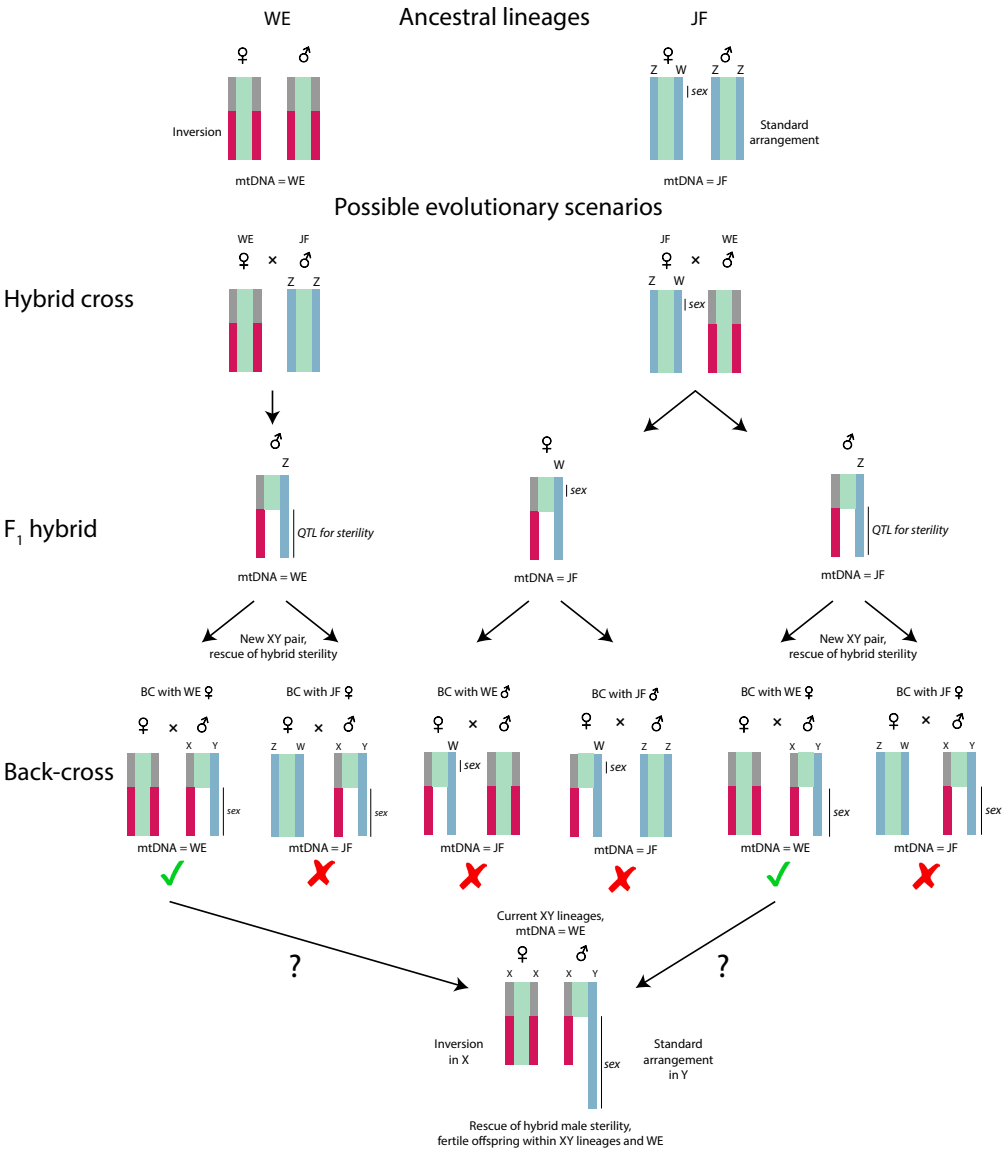


Figure 9. A schematic illustration of the formation of a new heteromorphic XY pair as observed in nine-spined sticklebacks. The ancestral JF lineage has a ZW sex chromosome system (blue), which recombines over most of its length. The JF and WE lineages are separated by an inversion (red). A new XY pair was formed by a hybridization event. Recombination between the new X chromosome, originating from the WE lineage, and the new Y chromosome from the JF lineage becomes restricted on the inversion region. Possible evolutionary scenarios for the formation of the new XY chromosome pair and rescue of hybrid male sterility are shown, two of which are considered as possible models based on the patterns of mtDNA data. The cytologically distinguishable Y chromosome has likely increased in size through the accumulation of repetitive DNA.

male offspring were found. A similar sex bias was observed in F_1 hybrids of female parents from the XY lineages and JF male parents. This bias is likely due to the lack of a dominant sex determiner in the hybrid crosses. Hybrid offspring of an XX female and ZZ male lack both presumably dominant Y- or W-linked sex determining genes, and their sex determination is based on X- and Z-linked alleles with recessive sex determination effects. This may result in male-biased sex ratios, which in turn may lead to the adoption of a new dominant sex determiner (Rubini *et al*. 1972, Kallman 1984, Ogata *et al*. 2003). A complete absence of females was observed in F_1 hybrids of WE females and JF males, which suggests that if sex in the WE lineage is based on a major sex-determining gene, it is likely male dominant. The Y chromosome of the XY lineages originates from the female heterogametic JF lineage, implying that a dominant male promoting sex-determining gene has hijacked the main sex-determining role on the chromosome of JF lineage origin. This may have happened when a new mutation obtained the primary sex-determining role, or when an ancestral dominant male-determining gene was translocated into the new proto-Y chromosome. Transitions between XY and ZW systems are possible if sex-specific chromosomes are not yet highly degenerated, and thus such transitions are only seen in taxa with young sex chromosomes that are in the early stage of their evolution (van Doorn and Kirkpatrick 2010).

Theoretical studies have suggested hypothetical factors driving transitions in heterogamety. In populations with biased sex ratios, a new sex determination system may be favoured by selection, and thus replace the ancestral sex chromosomes (Bull 1983). Empirical studies on transitions in heterogamety are scarce, but it has been suggested that a sex ratio bias resulted in a transition from female to male heterogamety in the Japanese frog Rana rugosa – a species with both XY and ZW systems (Ogata *et al*. 2003). Even though males of the XY lineages carry a chromosome pair originating from the WE and JF lineages, and thus the inversion region is expected to be heterozygous, they are fertile, suggesting that, whatever factor causes the male sterility of inversion heterozygotes in F_1 hybrids between ZW populations, it no longer acts in the XY lineages. Also, no hybrid male sterility was observed in hybrid families whose male parents are from the XY lineages and female parents from the WE lineage, demonstrating that despite the heterozygous inversion region of the offspring, the genomic conflict between the ancestral types of sex chromosomes causing male sterility in the F_1 hybrids has been recovered in the XY lineages. It is possible that the chromosome originating from the JF lineage has obtained the new male-determining role in the XY lineages and at the same time a mutation to rescue hybrid male fertility has emerged (Fig. 9). Studies on *Drosophila* suggest that such mutations may be mutant alleles of the loci that initially caused hybrid incompatibility (Barbash *et al*. 2000, Orr and Irving 2001).

Together, these results demonstrate the formation of different sex determination systems and postzygotic reproductive isolation in recently diverged lineages of the nine-spined stickleback, providing evidence for a chromosomal inversion leading to rapid sex chromosome evolution and reproductive isolation.

5. CONCLUSIONS AND FUTURE DIRECTIONS

In this thesis I have examined the striking variety in sex chromosome systems of closely related stickleback species. The three-spined stickleback is already considered a model species for sex chromosome evolution, and

the neo-sex chromosomes of the Japan Sea lineage can be particularly useful for further investigation of the early stages of sex chromosome differentiation. My findings on the intraspecific variation on sex chromosome systems and hybrid incompatibility in the nine-spined stickleback anticipate the emergence of this species as a new prominent model system for studying the evolution of sex determination and speciation.

The application of modern molecular genetic and genomic technologies has had and will continue to have a great impact on the field of evolutionary genetics and speciation genomics. Fast and affordable molecular methods make it easier to map sex-determining loci even in non-model organisms, and comparative studies on closely related species with different sex-determining systems may help us understand how and why transitions in sex determination evolve (Bachtrog *et al.* 2014). Genetic mapping and the characterization of genes and genomic regions restricting recombination and gene flow between hybridizing taxa in natural as well as experimentally generated hybrids is crucial for improving our knowledge of the occurrence and importance of different evolutionary processes in speciation (Noor and Feder 2006). Rapidly increasing empirical evidence supports the importance of genomic conflict in the evolution of reproductive isolation, but so far only a few studies have conclusively linked intragenomic conflict with speciation (Crespi and Nosil 2013). Future studies on speciation genomics will likely focus largely on the chronological order in which the genetic and phenotypic changes leading to speciation occur: to causally link a genomic conflict with speciation it must drive reproductive isolation, and thus occur before speciation is complete (Noor and Feder 2006, Crespi and Nosil 2013).

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